Isolation of Thymidine-resistant Cells from a Thymidine-sensitive Acute Lymphoblastic Leukemia Cell Line

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ABSTRACT

Malignant cells have enhanced sensitivity to inhibition of growth by thymidine. Cell growth of the permanent lymphoid cell line CCRF-CEM, originating from a patient with acute lymphoblastic leukemia, is inhibited by 3 x 10^{-5} \text{ M} thymidine, compared to 1 to 5 x 10^{-3} \text{ M} thymidine required to inhibit growth of normal lymphoid lines. Thymidine-resistant cells were isolated at a frequency of approximately 1/100,000 cells after cloning CCRF-CEM cells in medium containing 5 x 10^{-4} \text{ M} thymidine. The resistant cells lacked the enzyme thymidine kinase, had a 20-fold decrease of thymidine uptake, and were resistant to 1 x 10^{-4} \text{ M} 5-bromo-2-deoxyuridine. The cells were sensitive to 1 x 10^{-5} \text{ M} methotrexate, even in the presence of exogenously added thymidine and hypoxanthine. The data indicate that a small fraction of malignant cells may escape the toxic effect of high thymidine therapy and, therefore, require additional chemotherapy for their control.

INTRODUCTION

CCRF-CEM is a permanent lymphoid cell line established from a patient with ALL, which has a 20- to 100-fold greater sensitivity to growth inhibition by dThd than other mammalian cells in culture (14). dThd sensitivity has been reported in at least one other established ALL lymphoid cell line with a concomitant 10-fold decrease of dThd phosphorylase activity (8).

\[
\text{dThd} + P \rightarrow \text{dThd} + 2\text{-deoxy-d-ribose-1-phosphate}
\]

Lazarus et al. (8) postulated that the low level of dThd phosphorlase was related to the dThd sensitivity of ALL cells. Additional studies have documented that permanent ALL cell lines have metabolic and immunological differences from lymphoid lines established from donors without cancer (3, 9, 15). In the present study, a series of experiments was initiated with CCRF-CEM cells to select cells resistant to inhibition by high levels of dThd, and to determine the mechanism of their resistance.

MATERIALS AND METHODS

Cells were maintained as previously described (17). CCRF-CEM cells were cloned in soft agarose over a human diploid fibroblast feeder layer (13). The cells were cloned twice prior to the studies to eliminate preexisting cell variants. In one series of experiments, 500 of these cloned cells were placed in each of 15 microwells, grown to 4 x 10^6 cells, and immediately plated at a concentration of 2 x 10^6 cells/60-mm-diameter Petridish. The selective concentration of dThd was 5 x 10^{-4} \text{ M}. The cloning efficiency of cells in dThd-free media was approximately 40%. The cloning efficiency of dThd-resistant cells was not affected by the presence of 2 x 10^{-6} dThd-sensitive cells, as shown by a reconstruction experiment in which 100 dThd-resistant cells were plated in the presence of varying numbers of dThd-sensitive cells.

The [3H]dThd uptake studies were performed as follows. One x 10^6 cells were resuspended in 10 ml of Roswell Park Memorial Institute Medium 1640 and incubated at 37° with 5% CO_2:95% air for 2 hr. [3H]dThd (2 \mu Ci) was added aseptically. At designated times, 1-ml aliquots of cell suspension were removed and added to cold tubes containing 0.1 ml of 1 x 10^{-4} \text{ M} dThd. The tubes were centrifuged at 4°, and the cell pellet was washed twice in phosphate-buffered saline (136 mM, 0.8% NaCl, 2.7 mM KCl, 8.7 mM Na_2HPO_4, 0.84 mM KH_2PO_4, and 5.5 mM glucose) containing 1 x 10^{-5} \text{ M} dThd. The cells were collected on fiber glass filters and transferred to scintillation vials.

dThd phosphorylase (8) and dThd kinase (16) were assayed by published methods.

RESULTS

The number of dThd-resistant clones obtained with twice-cloned CCRF-CEM cells, after correction for a 40% cloning efficiency, was 121/6.6 x 10^4 cells, or 1/54,500 plated cells. The number of dThd-resistant clones in experiments initiated from 500 sensitive cells grown up to 4 x 10^6 cells before plating was 158/1.86 x 10^6 cells, or 1/118,000 plated cells.

Cell growth of the parent CCRF-CEM cells was completely inhibited at 5 x 10^{-5} \text{ M} dThd, while a typical resistant clone, CCRF-CEM-A10, was unaffected at that concentration and required 1 x 10^{-3} \text{ M} dThd for growth inhibition (Chart 1). The decreased sensitivity of the A-10 clone to dThd inhibition is similar to that of L-8, a lymphoid cell line established from a normal volunteer (Chart 1). The clones isolated from CCRF-CEM retained their resistance to dThd even after growth for 2 months in the absence of dThd.

The uptake of [3H]dThd by the resistant clones was reduced 20-fold compared to the parent CCRF-CEM cells (Chart 2). A slow but continuous uptake of [3H]dThd was observed in all resistant clones tested. The dThd analog, BrdUr, which is toxic to CCRF-CEM cells, had no effect on the resistant clones (Chart 3).

The level of dThd phosphorylase activity in both CCRF-CEM and resistant clones was absent, compared to normal lymphoid cell lines. However, dThd kinase activity was normal in CCRF-
Chart 1. Inhibition of cell growth by dThd of ALL cells (CCRF-CEM). ALL cells selected in 5 x 10^{-4} M dThd (CCRF-CEM-A10), and a normal lymphoid cell line (L-8).


DISCUSSION

Chromosomal damage has been observed in cells in which growth was inhibited by dThd (12). Therefore, it is necessary to consider possible chromosomal damage during the use of dThd to destroy malignant cells with enhanced sensitivity to dThd (6, 10). Since chromosomal damage was observed only at dThd concentrations which would normally inhibit DNA synthesis (12), such damage should be limited only to dThd-sensitive cells. However, the possibility of selection or induction of mutations in normal and malignant cell populations cannot be ruled out.

Isolation of rare mutations is made feasible by growing a large population of cells in an environment toxic to the majority of cells, while allowing the growth of rare cells. The dThd sensitivity of CCRF-CEM cells was used to eliminate the majority of the cells and selected for cells resistant to dThd. dThd, presumably after its conversion to TTP, inhibits the synthesis of phosphorylated derivatives of deoxycytidine, which are required for DNA synthesis (11).

Any mutation, spontaneous or induced, in dThd-sensitive cells can be divided into 2 phenotypic groups. The first would be sensitive to dThd, and the second would be resistant. The first group could theoretically contain an infinite variety of mutations, but since the cells would still be sensitive to dThd, the cells could not grow in the presence of dThd, although they might grow after the dThd therapy was terminated. The second group can be further subdivided into 3 subgroups. In the first subgroup are cells with an alteration in the basic mechanism responsible for the observed sensitivity of malignant cells to dThd (perhaps via a "derepression" of dThd phosphorylase activity). The absence of dThd phosphorylase activity in dThd-resistant cells (Table 2) eliminated this hypothesis. In the second subgroup are cells with an alteration in cellular permeability...
to dThd which would result in a resistant clone and is consistent with the decreased uptake of [3H]dThd (Chart 2) and resistance to BrdUrd (Chart 3). However, reduced uptake of dThd is also observed in cells with loss of dThd kinase activity (4, 5), the third subgroup. When both dThd kinase and dThd uptake are reduced, as in these studies (Table 2; Chart 2), it is assumed that the latter is a consequence of the loss of dThd kinase activity, rather than being the primary event (4). Therefore, the data suggest that the isolation of CCRF-CEM clones resistant to dThd is a consequence of loss of dThd kinase activity, rather than from an alteration of membrane permeability.

Cells lacking dThd kinase activity are usually selected because of their resistance to dThd analogs like BrdUrd or 5-iodo-2-deoxyuridine (4, 7). High levels of dThd have been used to select dThd kinase-deficient mutants in cultures of mouse lymphoma cells (1, 4), and the DON line of Chinese hamster fibroblasts (2). The dThd-resistant cells isolated in those studies retained up to 45% of the original dThd kinase activity, unlike the clones isolated from CCRF-CEM (Table 1). The appearance of resistant mouse lymphoma clones at a frequency of 1/28,700 cells plated (2) is comparable to the presently observed frequency of 1 in approximately 100,000 cells. The meaning of these high rates of spontaneous mutations have been discussed by Anderson and Fox (1).

The frequency of dThd-resistant cells is enhanced by pretreatment of cells with mutagens or X-rays (1). The frequency is also greater if 5-iodo-2-deoxyuridine is used as a selective agent rather than dThd (1). This has been interpreted as indicating that 5-iodo-2-deoxyuridine may be a mutagenic agent. The appearance of dThd-resistant cells has been assumed to be a spontaneous occurrence (1). The present data do not indicate whether the frequency of dThd kinase-deficient mutants is affected by the high levels of dThd. The data indicate that dThd permits the growth of malignant cells with a spontaneous mutation to dThd resistance. However, these cells retain their sensitivity to other chemotherapeutic drugs such as methotrexate (Table 1).

ACKNOWLEDGMENTS

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REFERENCES


Thymidine-resistant Cells Isolated from ALL Cells

Table 2

Effect of methotrexate on the growth of dThd-sensitive and -resistant cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Addition to media</th>
<th>Cells/ml x 10^6</th>
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<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 4</td>
</tr>
<tr>
<td>L-8</td>
<td>Normal</td>
<td>Methotrexate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methotrexate dThd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hypoxanthine</td>
</tr>
<tr>
<td>CCRF-CEM ALL</td>
<td>Methotrexate dThd</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>Methotrexate Hypoxanthine</td>
<td>4.0</td>
</tr>
<tr>
<td>CCRF-CEM-A10 dThd resistant</td>
<td>Methotrexate dThd</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>Hypoxanthine</td>
<td>4.0</td>
</tr>
</tbody>
</table>

* The cells were subcultured in triplicate in Roswell Park Memorial Institute Medium 1640 with 10% fetal calf serum. The additions, when present, were 1 x 10^-3 M methotrexate, 4 x 10^-3 M dThd, and 1 x 10^-4 M hypoxanthine, except for CCRF-CEM, where the concentration of dThd was 1 x 10^-4 M.

Table 1

<table>
<thead>
<tr>
<th>Cells</th>
<th>Origin</th>
<th>dThd phosphorylase* (μmol/hr/mg protein)</th>
<th>dThd kinase* (nmol/hr/mg protein)</th>
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</thead>
<tbody>
<tr>
<td>L-8</td>
<td>Normal</td>
<td>342</td>
<td>11.9</td>
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<tr>
<td>L-18</td>
<td>Normal</td>
<td>1033</td>
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<tr>
<td>L-55</td>
<td>Normal</td>
<td>734</td>
<td>10.2</td>
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<tr>
<td>L-5</td>
<td>Infectious mononucleosis</td>
<td>509</td>
<td>24.4</td>
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<tr>
<td>CCRF-CEM</td>
<td>ALL</td>
<td>0</td>
<td>20.0</td>
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<tr>
<td>CCRF-CEM-A2 dThd resistant</td>
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<td>0</td>
<td></td>
</tr>
<tr>
<td>CCRF-CEM-A4 dThd resistant</td>
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<td>0</td>
<td></td>
</tr>
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<td>CCRF-CEM-A6 dThd resistant</td>
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<td>0</td>
<td></td>
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<tr>
<td>CCRF-CEM-A8 dThd resistant</td>
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<td>0</td>
<td></td>
</tr>
<tr>
<td>CCRF-CEM-A10 dThd resistant</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* The limit of sensitivity of the dThd phosphorylase assay was ≤50 μmol/hr/mg protein.
* The limit of sensitivity of the dThd kinase assay was ≤0.2 nmol/hr/mg protein.


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